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SOME FACTORS INFLUENCING THE POTENCY OF CONCENTRATED ANTITOXIC SERUM

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The concentration of antidiphtheric serum has been carried on for a considerable period, but the process is still largely empirical as our knowledge of the chemistry of blood proteins is incomplete. The relation of antitoxic content to the blood protein is one of much interest both from a scientific and practical point of view, and any research which throws light on this question must be of value.

The first practical method of serum concentration was devised by Gibson[1] and consisted of precipitating the globulins of the blood serum or plasma by half saturation with ammonium sulphate solution and eliminating the albumin in the filtrate. The globulin precipitate, which is generally conceded to contain the bulk of antitoxin, was then dissolved in saturated sodium chlorid solution in order to further eliminate nonantitoxic protein, and the antitoxin globulins were precipitated from this solution with acetic acid. The precipitate was then gathered on hard filters, pressed and dialyzed against running water and the acid neutralized with sodium carbonate.

Banzhaf[2] modified the Gibson method as follows: The plasma was heated to 57 C. for from twelve to fifteen hours, cooled and diluted with half its volume of water and three parts saturated ammonium sulphate solution added to seven parts of diluted plasma. The precipitate was gathered on hard filters, pressed and dialyzed. By this method two serums were obtained, one of low and one of high potency.

In 1912 Banzhaf[3] published a further modification which consists of diluting the plasma with half its volume of water and adding enough saturated ammonium sulphate solution to make a 30% saturation. The mixture is heated to 60 C. in a water bath, and filtered while hot. The precipitate remaining on the filters is either washed with 33⅓% ammonium sulphate solution or is dissolved in water and ammonium sulphate solution added to bring the saturation to 33⅓%. The solution is filtered, the filtrate united with the first filtrate and the whole brought to 50% saturation with ammonium sulphate solution. The resulting precipitate is pressed and dialyzed as in the previous method. Later in 1915 Banzhaf[4] recommended holding the plasma ammonium sulphate mixture at 60 C. for 15 minutes. Banzhaf has since advocated the addition of 0.25 to 0.3% phenol to the plasma as a preservative. The added phenol tends to prevent clotting of the plasma and also gives a higher concentration of the finished product.

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[1] Jour. Biol. Chem., 1906, 1, p. 161.

[2] Collected Studies from the Research Laboratory, Dept. of Health, City of New York, 1908-9, 4, p. 230.

[3] Ibid., 1912-13, 7, p. 114.

[4] A report presented by the Committee on Standard Preparation of Diphtheria Antitoxin to the American Public Health Association in September, 1915.

Homer[5] advocated the following modifications of the Banzhaf methods:

"The serum or plasma, diluted with one-third or even with only one-fifth its volume of water, and made 1.5 to 2% with solid sodium chlorid, is heated to a temperature of 56-57 C. for 15 hours or to 57-58 C. for 8 hours, stage 1.

"The heated plasma is then made 30% of saturation with ammonium sulphate and the mixture is heated to a temperature of 61 C. and kept at that temperature for a few minutes only, stage 2.

"The mixture after cooling to 40-45 C. is filtered. The precipitate is washed with 33% of saturation with ammonium sulphate. The washings, after being filtered, are added to the main bulk of the filtrate which is then made 50% of saturation with ammonium sulphate.

"The resulting precipitate is filtered off, pressed and dialyzed. The pressed precipitate has a yellowish color and not the bluish green color of Banzhaf's product."

Homer[6] stated that before heating, the reaction of the plasma should be adjusted to about P_H 8 or that 0.25 and not more than 0.30% trikresol should be added.

The relation of antitoxin to the proteins of the blood is not fully understood. It has been demonstrated by Pick[7] and others that antitoxin is associated with the pseudoglobulin fraction of the blood proteins. However, little is known as to the nature of this association.

The experiments reported in this paper were designed to investigate the influence of heat on the antitoxic content of the globulin fraction, and are considered under two heads, namely:

(A). Determination of the effect of heat on antitoxic plasma, by the application of increasingly greater amounts of heat to the plasma ammonium sulphate mixture.

(B). Determination of the changes that have been produced in the dialyzed products by fractioning.

The blood proteins are usually divided into three more or less clearly defined fractions, according to their property of being precipitated by various salts.

Experience has shown that a variable portion of antitoxin is precipitated by 30-38% saturation with ammonium sulphate solution. It is therefore expedient for economic reasons to preserve the globulin precipitated with ammonium sulphate in saturation from 30 to 50%, otherwise the loss would be too great when antitoxin is concentrated for commercial purposes.

The precipitability of the different proteins in the plasma depends on the influence of several factors, namely, the presence of different salts, the degree of saturation with ammonium sulphate, the presence of phenol compounds, the amount of heat applied, the reaction of the plasma, and possibly other factors. For example, trikresol or phenol when added to the plasma before concentration tends to alter the precipitating limits of the protein and gives a higher degree of concentration in, the finished product. For this reason phenol compounds, were purposely omitted from the experiments, although the final product gains in degree of concentration and in attractiveness. as has been shown by Heineman's[8] method of reconcentration.

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[5] Jour. Hyg., 1916, 15, p. 388.

[6] Jour. Hyg., 1918, 17, p. 51.

[7] Beitr. z. chem. physiol., 1901-2, 1, p. 351.

The method used in concentrating the serums for the experimental work incorporated in this paper was the Banzhaf method modified by Heineman, excepting that different portions of the plasma were heated to 60, 64, 68 and 72 C., respectively. The first steps were the same as in the Banzhaf process except that the plasma ammonium sulphate mixture was heated for one and three quarter hours to reach the desired temperature, held at that temperature for one half hour, and filtered while hot. The resulting precipitate was stirred in water in order to separate the soluble from the insoluble globulins, and after the lapse of one hour, enough ammonium sulphate solution was added to equal one half of the amount of water used, thus making a 33⅓% saturation. The antitoxin globulins remain in solution while the fibrinoglobulin is precipitated and separated by filtration. The precipitate on the filters is washed twice with 33⅓% ammonium sulphate solution to recover the dissolved antitoxic globulins.

The resulting filtrate was added to the first filtrate and enough saturated ammonium sulphate solution added to make a 50% saturation. The precipitate was gathered on soft filters and stirred in a measured volume of water. After the solution was completed it was strained through cheese-cloth and the remaining paper pulp washed twice with water. To this solution a volume of saturated ammonium sulphate, equal to the total amount of water used for solution, was added and the precipitate gathered on soft filters. The process of redissolving and reprecipitating was repeated until the filtrate was colorless and clear. Usually two solutions were sufficient to accomplish this end so that finally the globulin precipitate could be gathered on hard filters. The precipitated globulins usually showed a greenish color. The precipitate was pressed and dialyzed in the usual manner.

(A) DETERMINATION OF EFFECT OF HEAT ON ANTITOXIC PLASMA BY THE APPLICATION OF INCREASINGLY GREATER AMOUNTS OF HEAT TO THE PLASMA AMMONIUM SULPHATE MIXTURE

Forty liters of each diphtheria and tetanus plasma containing chloroform as a preservative were divided into 4 equal portions, each of which was heated to 60, 64, 68 and 72 C., respectively. The solids and potency[9] of each plasma were determined previous to heating.

The diphtheria plasma used had aged for three months. It was a mixture of several bleedings from two different horses, and was filtered through paper before further treatment was undertaken. Potency tests showed the mixture to contain 150 units per c c and 9.060% solids. One gram solids, therefore, contained 1,655 units diphtheria antitoxin.

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[8] Jour. Infect. Dis., 1916, 19, p. 433.

[9] Potency tests were conducted in accordance with the rule laid down by the Hygienic Laboratory. No tests were taken as final unless the control guinea-pig died promptly in three days or somewhat less, and the final tests were based on the guinea-pig which survived 96 hours.

TABLE 1

Results of Concentrating 10,000 c c Quantities of Diphtheria Plasma at Various Degrees of Temperature

Degree of Heat Applied	Volume Obtained, c c	Total Number of Units Obtained	Percentage of Loss of Units	Percentage of Units Recovered	Percentage of Units Theoretically Lost but Recovered in the Soluble Globulin	Units per c c	Multiple of Concentration	Units per c c *	Multiple of Concentration*	Percentage of Solids	Solids Recovered, Gm.	Percentage of Solids Recovered	Percentage of Loss of Solids	Units in 1 gm. Solids	Percentage of Increase in Units per Gram Solids
60	1,200	1,260,000	16.0	84.0	39.6	1,050	7.0	625	4.1	33.54	402.480	44.4	55.6	3,130	89.12
64	840	1,134,000	24.4	75.6	46.1	1,350	9.0	850	5.6	31.74	266.616	29.5	70.5	4,253	156.98
68	570	712,500	52.5	47.5	32.0	1,250	8.3	1,017	6.7	24.58	140.106	15.5	84.5	5,085	207.25
72	230	241,500	83.9	16.1	10.0	1,050	7.0	930	6.2	22.60	51.980	6.1	93.9	4,646	206.46

A study of table 1 brings out several important facts. The solids recovered decrease progressively as the temperature applied increases, but the potency per gram of solids increases as the temperature advances to 68 C. However, the potency of the solids obtained after heating to 72 C. is less than that of the solids heated to 68 degrees, although greater than that of the solids heated to 60 and 64 degrees. It is seen, therefore, that an increase in temperature for heating the plasma ammonium sulphate mixture eliminates a progressively greater amount of protein, while the potency of the dry globulins increases to a marked degree. The multiple of concentration is greatest at 68 C. Chart 1 shows the ratio of per cent. solids recovered to per cent. units recovered.

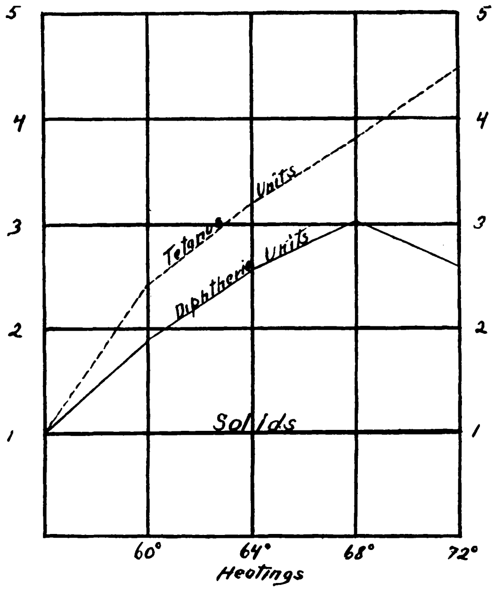

Chart 1.—Ratio of percentage of solids recovered to units recovered in both the tetanus and diphtheria concentrations.

The total quantity of antitoxin lost by increase of temperature is so great as to render heating higher than 60 C. prohibitive from a commercial point of view. However, the interesting fact remains that with the elimination of protein a considerable amount of antitoxin is transferred from the globulin fraction which has been rendered insoluble by heat to the more soluble fraction. This principle holds throughout the heatings and is greatest at 64 C.

A preliminary series of experiments was conducted in which a portion of serum was heated to 76 C. The remaining antitoxin was so small that it seemed unprofitable to use a temperature higher than 72 C.

The relation of the loss of protein to the loss of antitoxin appears clearly from the following considerations: The loss of protein resulting from the heating to 64 C. is greater by 33.75% than the loss at 60 C., while the loss of antitoxin is only 10%. The loss of protein is 135.837 gm., representing a loss of 425,167 units if the antitoxin content of 3,130 units per gram solids of the 60 C. heating is taken as a basis for calculation. However, the actual loss in antitoxic units caused by heating to 64 degrees is greater by 126,000 units than the loss by heating to 60 C. The difference between the theoretical loss of 425,169 units and the actual loss of 126,000 units is 299,169 units, which were not lost in the precipitated protein but remained in the soluble portion.

Comparison of the results of the 68 C. heating with those of the 64 C. heating brings out similar relations. The globulin contained in the final product of the 68 C. heating is 126.510 gm. less than that contained in the 64 C. heating. This represents a loss of 538,047 units antitoxin if calculated on the value of the dialyzed product of the 64 C. heating, namely, 4,253 units per gram solids. The actual loss, however, was 421,500 units, leaving a difference of 116,547 units which were retained in the soluble portion of the globulin.

If the results of the 72 C. heating are compared with those of the 60 and 64 degree heatings, similar conditions present themselves, but when comparing the 72 C. heating with the 68 degree heating, it is found that there is no antitoxin transferred to the soluble globulin fraction.

(B) DETERMINATION OF CHANGES PRODUCED IN THE DIALYZED PRODUCTS BY FRACTIONING

To 200 c c of the diluted serum, which was equivalent to 100 c c of the original dialyzed product, was added enough distilled water to bring the solids to approximately 10%. This diluted serum was made to 38% saturation by the addition of saturated ammonium sulphate solution. After standing for about one hour it was placed on a hard filter, and the precipitate was washed twice with 38% ammonium sulphate solution and then pressed and dialyzed.

The filtrate was made to 50% saturation by adding saturated ammonium sulphate solution. This mixture stood for about an hour when it was placed on a hard filter. The gathered precipitate was pressed and dialyzed.

TABLE 2

RESULTS OF FRACTIONATING 100 C C OF THE DIALYZED PRODUCTS OF THE FOUR HEATINGS

Degree of Heat Applied	Units per 1 c c	Volume of Dialized Product	Fractionated 100 c c							Units Recovered	Units Lost
				38%			50%				
			Percentage of Solids	Solids	Entire Units	Units per Gram Solids	Solids	Entire Units	Units per Gram Solids		
60	1,050	1,200	33.540	29.206	93,500	3,201	1.890	8,250	4,365	101,750	3,250
64	1,350	840	31.740	25.380	85,500	3,368	3.680	18,750	5,095	104,250	30,750
68	1,250	570	24.580	19.123	84,500	4,418	3.744	19,500	5,208	104,000	21,000
68*	1,250	570	24.580	11.637	36,000	3,093	9.918	58,500	5,898	94,500	30,500
72	1.050	230	22.600	18.058	84,500	4,679	2.135	10,000	4,683	94,500	10,500

* Fractionated without the addition of trikresol or NaCl.

The percentage of solids in the dialyzed products of the various heatings vary to such an extent that, in order to compare the results of fractionating, the data of Table 2 were reduced to a basis of 20% solids and incorporated in Table 3.

TABLE 3

RESULTS OF FRACTIONATING REDUCED TO A BASIS OF 20% SOLIDS

Degree of Heat Applied	Fractionated 100 c c						
	38%		50%		Units Recovered	Units Lost	Solids Lost
	Solids	Units	Solids	Units			
60	17.415	55,745	1.127	4,919	60,664	1,836	1,458
64	15.992	53,861	2.318	11,810	65,671	19,330	1.690
68	15.543	68,669	3.046	15,864	84,533	17,127	1.411
68*	9,468	29,284	8.069	47,590	76,874	24,826	2.462
72	15.980	74,770	1.889	8,846	83,616	9,384	2.131

* Fractionated without the addition of trikresol or NaCl.

Table 3 shows a gradual increase of solids in the 50% fraction up to the 68 C. heating accompanied by an increase in units per gram solids. The unfractioned serums and their 38% fractions are lower in units per gram solids than their corresponding 50% fractions. After heating to 72 C. the solids of the 50% fraction decrease as do the units per gram solids.

The number of units lost in fractionating the serum prepared at 60 C. is small when compared with the units lost in fractionating serums prepared at higher temperature. The number of units lost in fractionating increases markedly as the temperature applied increases, while the amount of solids lost is not great.

The relation of the loss of protein to the loss of antitoxin is clearly shown by the following data: The solids lost in fractionating the serum of the 60 C. heating were 1.458 gm., which are equivalent to 4,564 units antitoxin if the potency of 1 gm. solids of the unfractionated product is taken as a basis of calculation. The actual loss of antitoxin was 1,836 units. The difference between the theoretical loss of 4,564 and the actual loss of 1,836 units is 2,728 units which were not lost with the protein but remained in the more soluble fraction. A small portion of the protein was lost during the manipulation, but since the proportionate amount of antitoxin was not lost an explanation must be looked for elsewhere. Possibly the added trikresol converted a sufficient quantity of nonantitoxic protein into an insoluble form so that loss of protein by precipitation and subsequent filtration and retention of antitoxin in solution resulted.

In fractionating the serums resulting from the 64, 68 and 72 degree heatings, the reverse was true. The number of units actually lost in fractionating exceeds the number of units represented by the solids lost. It is clear that in these cases the presence of trikresol is not the only factor that operates to increase the loss of both solids and antitoxin. Probably the higher degree of heat applied has so affected the antitoxic proteins that they are more readily precipitated by ammonium sulphate than the antitoxic proteins which are contained in the products resulting from the 60 degree heating. Therefore at degrees of temperature above 60 the actual loss of protein in fractionating is not materially different from the loss at 60 degrees, but the loss of antitoxin is decidedly greater. The protein rendered insoluble by heating to 60 C. of course is not contained in the dialyzed product used for fractionating as it was removed by filtration of the original heated plasma ammonium sulphate mixture.

In fractionating the 72 degree heating, the actual loss in units is represented by the solids lost in fractionating. This fact bears out the theory developed in the previous paragraph. Heating to 72 C. brings all the globulin so near the critical point of complete precipitation that but small quantities of both globulins and antitoxin remain.

The relation between the loss of solids and antitoxin in fractionating serums prepared at temperatures above 60 C. is shown by the fractionated 64 degree heating. The solids lost in fractionating were 1.690 gm., which are equivalent to 7,188 units if the units per gram solids of the unfractioned 64 degree heating are taken as a basis of calculation. The actual loss of antitoxin was 19,330 units. The difference between the theoretical loss of 7,188 units and the actual loss of 19,330 is 12,142 units.

The units per gram solids of the 38% fractions are lower than those of the corresponding unfractioned serums with the exception of the 60 and 72 degree heatings. In these the units per gram solids are practically the same as those in the unfractioned serum. The 50% fractions are considerably higher in units per gram protein than either those of the unfractioned serums or those of the 38% fractions, except in the 72 degree heating where the unfractioned and the 38 and 50% fractions each contain practically the same number of units per gram protein.

The tetanus plasma used had aged for two years and was prepared for concentration in the same manner as the diphtheria plasma. Potency tests showed the combined 40 liters of plasma to contain 45 units per c c and 8.570% solids. One gram of solids therefore contained 525 units tetanus antitoxin.

(A) Determination of the effect of heat on antitoxic plasma, by the application of increasingly greater amounts of heat to the plasma ammonium sulphate mixture.

TABLE 4

Results of Concentrating 10,000 c c Quantities of Tetanus Plasma at Various Degrees of Temperature

Degree of Heat Applied	Volume Obtained, c c	Total Number of Units Obtained	Percentage of Loss of Units	Percentage of Units Recovered	Percentage of Units Theoretically Lost but Recovered in the Soluble Globulin	Units per c c	Multiple of Concentration	Units per c c *	Multiple of Concentration*	Percentage of Solids	Solids Recovered, Gm.	Percentage of Solids Recovered	Percentage of Loss of Solids	Units in 1 gm. Solids	Percentage of Increase in Units per Gram Solids
60	1,080	378,000	16.00	84.00	49.06	350	7.7	252	5.6	27.72	299.376	34.94	65.06	1,262	140.4
64	700	350,000	22.22	77.78	53.68	500	11.1	338	7.5	29.50	206.500	24.10	75.90	1,695	222.8
68	390	214,000	52.44	47.56	35.27	550	12.2	406	9.0	27.00	105.300	12.29	87.71	2,037	228.0
72	230	149,500	66.77	33.23	25.91	650	14.4	477	10.6	27.24	62.652	7.32	92.68	2,386	354.4

A study of table 4 shows a similarity of results with those of the diphtheria concentrations. The progressive decrease in solids recovered, as the temperature applied increased, was somewhat greater than in the diphtheria concentrations with the exception of the 72 C. heating in which the solids recovered were proportionately greater than in the diphtheria heating. The potency per gram solids increased throughout the heatings, while in the diphtheria serums the highest unitage per gram protein was reached at 68 C. The multiple of concentration increased markedly as the temperature applied increased. The highest point was reached at 72 degrees, where the concentration of units per cubic centimeter was 14.4 times that of the plasma. The highest multiple of concentration in the diphtheria serums was at 68 degrees. See chart 1 for a comparative ratio of percentage of solids recovered to percentage of units recovered by both diphtheria and tetanus concentrations.

Throughout the heatings antitoxin was transferred from the globulin fraction, which was rendered insoluble by heat, to the more soluble fraction. The greatest percentage transfer of units occurred at 64 degrees, as was the case in the diphtheria concentrations. There was a greater percentage recovery of units and a decrease in percentage recovery of solids in the tetanus concentrations over the diphtheria concentrations.

The relation of the loss of protein to the loss of antitoxin was similar to that of the diphtheria heatings, except that the percentage transfer of units from the protein made insoluble by heat to the more soluble globulins was greater than in the diphtheria serums. This applies to all the heatings and to comparisons between the different heatings. There was no transfer of antitoxin to the soluble globulin fractions when comparing the loss of protein to the loss of antitoxin between the 72 and the 68 degree heatings of the diphtheria concentrations.

The percentage increase in units per gram solids increased markedly as the temperature applied increased and was much greater at 72 degrees than in the corresponding diphtheria heating.

Determination of the Changes that Have Been Produced in the Dialyzed Products by Fractionating

The same method was used in fractionating the tetanus serums as was used in fractionating the diphtheria serums.

TABLE 5

Results of Fractionating 100 c c of the Concentrated Tetanus Serums of the Four Heatings

Degree of Heat Applied	Units per 1 c c	Units per Gram Solids	Fractionated 100 c c							Units Recovered	Units Lost
				38%			50%				
			Percentage of Solids	Solids	Entire Units	Units per Gram Solids	Solids	Entire Units	Units per Gram Solids		
60	350	1,262	27.72	23.148	28,800	1,244	3.220	6,125	1,902	34,925	75
64	500	1,695	29.50	24.776	40,500	1,634	2.758	8,750	3,172	49,250	750
68	550	2,037	27.00	21.568	40,000	1,854	2.765	10,150	3,670	50,150	4,850
68*	550	2,037	27.00	16.969	......	.....	7.296	22,500	3,083		
72	650	2,386	27.24	20.143	52,500	2,606	3.780	12,250	3,240	64,750	250

* Fractionated without the addition of trikresol or NaCl.

The data of table 5 were reduced to a basis of 20% solids in order to compare better the results of fractioning, and were incorporated in table 6.

TABLE 6

RESULTS OF FRACTIONATING REDUCED TO A BASIS OF 20% SOLIDS

Degree of Heat Applied	Fractionated 100 c c						
	38%		50%		Units Recovered	Units Lost	Solids Lost
	Solids	Units	Solids	Units			
60	16.701	20,776	2.323	4,418	25,194	46	0.976
64	16.797	27,446	1.870	5,931	33,377	423	1.333
68	15.976	29,619	2.048	7,516	37,135	3,465	1.976
68	12.569	......	5.404	16,760	......	.....	2.027
72	14.789	38,540	2.738	8,871	47,411	289	2.473

* Fractionated without the addition of trikresol or NaCl.

There was no progressive rise or fall in the gram solids of the 50% fractions, but there was a decided increase in units per gram solids both of the 38 and 50% fractions, as the temperature applied increased. The 38% fractions corresponded closely in units per gram solids to the units in the unfractioned serums, while the potency of the 50% fractions showed a marked increase over that of the unfractioned serums and the 38% fractions. This difference became less marked in the 72 C. heating. It is possible that the aging of the plasma caused a change in the proteins so that on concentration a greater proportion of the less soluble or nonantitoxic protein was eliminated throughout all the heatings. Consequently, the end-products of the different heatings contained less of the less soluble globulins and a greater multiple of concentration resulted than in the diphtheria serums which were aged for three months only. In the fractionated diphtheria serums the 38% fractions of the 64 and 68 C. heatings were less in units per gram solids than the unfractioned serums.

In fractionating the serums resulting from the various heatings, the number of units actually lost is less than the number of units represented by the solids lost in fractionating. The reverse was true in the diphtheria heatings with the exception of the 60 degree heating. Perhaps the addition of trikresol to the globulin solutions of the tetanus heatings did not cause as marked a change in the proteins as was experienced with the diphtheria heatings, for the reason that the globulin of the tetanus heatings was more soluble than the corresponding diphtheria globulin. That so great a change was not experienced is seen by the results of fractionating both diphtheria and tetanus serums without trikresol and comparing them with the same serums fractionated with trikresol. In the diphtheria serums (see table 3) when fractionated without trikresol the ratio of the solids of the 38% and the 50% fractions is 1.18 to 1, while the same serum fractionated with trikresol is 5.1 to 1, indicating that the precipitating limits of the proteins have been so altered by the addition of trikresol that they are capable of precipitation at a lower saturation with ammonium sulphate. In the tetanus serums (see table 6) when fractionated without trikresol the ratio of solids in the 38 and 50% fractions is 2.3 to 1, while the same serum with trikresol added is 7.8 to 1. It is seen that the addition of trikresol to the tetanus serum did not cause as great a change in the protein as did the addition of trikresol to the diphtheria serums, for the reason that the diphtheria serums contained proportionately more of the less soluble globulins than did the tetanus serums.

The tendency of the 38 and 50% fractions of the 72 C. heatings of both diphtheria and tetanus serums to give a unitage per gram protein approaching that of the unfractioned serums, can be explained on the assumption that at this temperature the globulins have closely approached the point of complete precipitation, and that the remaining globulins are of the more soluble type. Therefore, when fractionated, the fractions will not be so materially different from the unfractioned serums both in solubility of globulins and in unitage per gram protein.

SUMMARY

The physical condition of refined serums is not at all constant, a fact which is disturbing and puzzling to those engaged in the production of antitoxins. Uniformity in color, clarity, viscosity, relative potency, and keeping qualities of different products, has so far not been successful. The desirability of obtaining uniform serums is obvious, and it was one of the problems underlying this research to attempt to locate the causes of this variability.

There are several factors which seem to be influential in determining the color of the finished product. Fresh plasma when concentrated has a decidedly greenish tint, but as the ripening process of plasma is lengthened the green color becomes less apparent. The diphtheria and tetanus plasmas used in these experiments had aged three months and two years, respectively, and were concentrated under otherwise identical conditions. The concentrated diphtheria serum had a decidedly greenish tint, while the concentrated tetanus serum was of a yellowish color, and no trace of green remained. Whether the addition of chloroform as a preservative has an influence on the color is problematical. It is certain, however, that when some kinds of trikresol are used in place of chloroform no green color appears.

Serums, concentrated by the method used in this work in which the precipitated globulins were dissolved and precipitated repeatedly, were greenish, while the aged products were light yellow. This change of green to yellow in most cases was not accompanied by a precipitate.

The degree of temperature applied to the plasma ammonium sulphate mixture seems to influence the color of the dialyzed product and also its clarity. As the temperature applied increased the color of the end-product was gradually obscured by the formation of a colloidal precipitate, as the critical point for complete precipitation of the globulins was approached. This precipitate was most marked at 72 C. After standing in the refrigerator for three months a marked precipitate settled in the serum prepared at the higher temperature, but none was perceptible in that of the lower heatings. The green color that was so marked in the diphtheria serums entirely disappeared after seven months.

An interesting point was observed in the color of the fractions of the different serums. The 38% fractions had the same color throughout the heatings as the unfractioned serums, while the 50% fractions were water clear and had a decidedly green tint. Even the tetanus serums showed a trace of green in the 50% fractions, but this color was lost after a few weeks' standing. The green color gradually changed to yellow after six month's standing in the refrigerator at a low temperature.

These facts indicate that the green color of serums is associated with the more soluble fractions of the globulins, and that it is not stable, but changes to a yellowish color on standing. The addition of either NaCl or trikresol to the plasma previous to concentration seems to produce a yellowish serum, and it is possible that NaCl or trikresol when added to a finished product with a greenish tint aids in the change from green to yellow.

Mention has been made of the fact that the degree of heat applied has some influence on the clarity of serums. This is true especially when the point of complete precipitation of the globulins is approached. It was found that by heating to as high a temperature as 72 C., a colloidal precipitate was formed that was not removed by filtration, but that after standing at a low temperature for several months it settled to a marked degree.

Some authors have stated that the addition of trikresol to the plasma before concentration gives a clear end-product with a high multiple of concentration. Different serums react differently in regard to the formation of an insoluble precipitate when trikresol is added to the dialyzed product and the kind of trikresol used has some bearing on this point. Serums that are concentrated with the addition of trikresol to the plasma, as well as those that are concentrated with trikresol, are able to form a precipitate after trikresol has been added to the final product. The end-products of the tetanus were of a clear, yellowish-brown color when removed from the dialyzing bags, but on the addition of trikresol a cloudiness formed which was not diminished after eight months' standing. The diphtheria serums did not react this way even though the same trikresol was used for both. Homer[10] states that the reaction of the plasma is of importance in gaining a clear end-product. The addition of trikresol to the plasma or the adjusting of the reaction seems to give a clear end-product in some but not in all cases.

Plasma from certain horses will usually give a cloudy end-product when the usual methods of concentration are used. If the concentration of ammonium sulphate is increased from 30 to 33%, a clear product can be obtained in most cases. This statement agrees with Homer's[11] findings.

The solubility of the globulins in different concentrated serums is not the same. Globulins precipitated between 30-38% tend to produce a cloudy serum and are less soluble than those precipitated between 38-50%, which usually yield a clear solution. The 38% fractions have the same clarity as the unfractioned serums. The addition of trikresol evidently so alters the precipitating limits of the globulins that the less soluble ones are precipitated at a lower saturation than they would be without trikresol. The more soluble globulins form a clearer solution when trikresol is added than without this addition.

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[10] Biochem. Jour., 1917, 11, p. 21.

[11] Ibid., 1919, 13, p. 45.

In fractionating the concentrated serums without the addition of trikresol no precipitate was formed on making to 28% saturation with ammonium sulphate solution. However, after trikresol was added and the serums were aged for several days, a precipitate was formed on making to 28% saturation with ammonium sulphate. After dialysis this precipitate was but slightly soluble. The results obtained from serums containing trikresol were so at variance with those obtained from serums without trikresol that it was deemed best to reserve this subject for future study (see tables 3 and 4).

The viscosity of the various serums became less as the temperature applied increased and was not in direct proportion to the solid content of the various serums. In the concentrated diphtheria serums the percentage of solids decreased with a decrease in viscosity, but in the concentrated tetanus serums the percentage of solids did not vary to any great extent. However, the potency per gram solids of the viscous serums increased with a decrease in viscosity. As the temperature applied increased, an increasingly greater amount of protein was eliminated so that the globulins in the lower heatings contained more of the less soluble globulins than in higher heatings. The pressed globulin precipitates of the lower heatings, which gave viscous serums, were gummy. As the temperature applied increased, this property was gradually lost and the pressed precipitates became friable.

The 38% fractions were less soluble than the 50% fractions. The latter were friable and highly soluble. The potencies of the 38% fractions were lower than those of the 50% fractions per gram solids, and as the viscosity decreased the potency per gram solids of both fractions increased. Banzhaf and Gibson[12] also found that the more soluble portions of the "pseudoglobulin" are richest in antitoxin per gram protein.

As the temperature of the heatings increased, the percentage loss of protein and antitoxin increased, and after heating at 72 C. the protein rendered insoluble by heat and ammonium sulphate in the diphtheria plasma reached 94% with an accompanying loss of 84% of antitoxin, while in the tetanus plasma the percentage of loss of protein was 93 with 67% loss of antitoxin. In all of the heatings the percentage loss of protein was greater than the corresponding percentage loss of antitoxin. With an increase of temperature the multiple of concentration and units per gram solids were also increased, and at the higher heatings the percentage increase in units per gram solids over the plasma used was around 207% in the diphtheria concentrations and 354% in the tetanus concentrations. The relative potency of the plasma is a factor in determining the final multiple of concentration. It was noted that in this experimental work, as well as in the routine concentration of serums, plasma of low potency yields a greater multiple of concentration than plasma of high potency.

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[12] Jour. Biol. Chem., 1907, 3, p. 254.

In the fractioned serums of both diphtheria and tetanus, the 50% fractions contained more units per gram protein than the 38%, or the unfractioned serums, and as the temperature increased the units of the 50% fractions also increased. The 50% fractions of the tetanus serums showed a greater proportionate increase in units per gram protein over the unfractioned serums than did the 50% fractions of the diphtheria serums. The multiple of concentration was also greater than that in the diphtheria serums. This can be explained by the assumption that as the tetanus plasma had aged for a much longer period than the diphtheria plasma, a greater change had taken place in its proteins. In the aging of the plasma the proteins were so altered that on precipitation with 30% ammonium sulphate and heat a greater proportion of the less soluble globulins was removed than was the case with the diphtheria serum. This explanation is supported by the fact that the viscosity of the tetanus serums throughout all the heatings was less than the viscosity of the corresponding diphtheria serums. Furthermore, the units per gram protein of the 38% fraction of the tetanus serums approached closely the unitage of the unfractioned serums, while the unitage per gram protein of the 38% fraction of the diphtheria serums was smaller than the units per gram protein of the unfractioned serums with the exception of the 60 and 72 degree heatings.

Since the difference in unitage between the 38% fractions and the unfractioned serums of the tetanus serums is smaller than the difference between the 38% fraction and the unfractioned diphtheria serum, it seems probable that through prolonged aging the less soluble portion of globulin is so altered as to facilitate its removal by precipitation.

CONCLUSIONS

The color of concentrated serums is a factor determined by the age of the plasma previous to its concentration, by the method used in concentration, and by the presence of certain chemicals used as preservatives.

Serums concentrated by the method used in this work are green, but as the period of aging of the plasma is lengthened previous to concentration, the intensity of the green color gradually diminishes, and finally a product with a yellowish color remains.

The green color is associated chiefly with the more soluble portions of the pseudoglobulin.

The addition of trikresol to plasma previous to concentration causes the end-product to be of yellowish color with no trace of green.

When plasma is aged previous to concentration the proteins are so altered that a greater proportion of the nonantitoxic proteins is eliminated in concentrating than when plasma is not aged. The addition of trikresol to the fresh plasma previous to concentration seems to alter the proteins of the plasma in a similar way as does the process of aging.

The aging of the plasma or the addition of trikresol to the plasma previous to concentration lessens the viscosity of the finished product owing to the increased elimination of nonantitoxic proteins, which are of a less soluble nature, and a higher multiple of concentration is obtained.

The multiple of concentration depends in a measure on the potency of the plasma concentrated. It has been noted that the multiple of concentration is greater when low-unit plasma is concentrated than when high-unit plasma is concentrated.

The percentage precipitation of protein from heated plasma ammonium sulphate mixtures containing 30% ammonium sulphate solution is increased as the temperature applied is increased and is not complete at 76 C.

With the increased elimination of protein an increased percentage of antitoxin is also precipitated, but the percentage loss of antitoxin is less than the percentage loss of protein.

With the increased elimination of protein as the temperature applied is increased, there is a transfer of antitoxin from the protein rendered insoluble by heat and ammonium sulphate to the more soluble fraction. This principle is not lost even when the critical point for complete precipitation of the proteins is closely approached.

As the temperature is increased in concentrating, the potency of the end-products obtained becomes greater as also do the units per gram solids. However, heating higher than 60 C. for one half hour is not advisable owing to an appreciable loss of antitoxin.

The more soluble portion of the pseudoglobulin fraction is proportionately richer in antitoxin than the less soluble portion.

With the application of increasingly greater amounts of heat the ratio of solids of the more soluble to those of the less soluble fractions of the pseudoglobulin is not materially changed, but the potency of both fractions of the pseudoglobulins is increased.

As the temperature applied reaches a point near the complete precipitation of the globulins, the character of the end-product obtained is that of the more soluble fraction of the pseudoglobulin.